CYCLIC NUCLEOTIDE PHOSPHODIESTERASES

This application is a continuation of U.S. Patent Application Serial No. 09/454,060, filed December 2, 1999, which is a divisional application of U.S. Patent Application Serial No. 09/255,748, filed February 23, 1999, now U.S. Patent No. 6,080,548, issued June 27, 2000, which is a divisional application of U.S. Patent Application Serial No. 08/974,565, filed November 19, 1997, now U.S. Patent No. 5,932,423, issued August 3, 1999, which is a continuation-in-part application of U.S. Patent Application Serial No. 08/624,663, filed March 25, 1996, now U.S. Patent No. 5,798,246, issued August 25, 1998, all entitled "Novel Cyclic Nucleotide Phosphodiesterases," the contents of which are expressly incorporated in their entirety herein by reference.

FIELD OF THE INVENTION

This invention relates to the nucleic acid and amino acid sequences of cyclic nucleotide phosphodiesterases and to the use of these sequences in the diagnosis, prevention, and treatment of cancer and immune and neuronal disorders.

BACKGROUND OF THE INVENTION

Cyclic nucleotides (cAMP and cGMP) function as intracellular second messengers to transduce a variety of extracellular signals, including hormones, light, and neurotransmitters. Cyclic nucleotide phosphodiesterases (PDEs) degrade cyclic nucleotides to the corresponding monophosphates, thereby regulating the intracellular concentrations of cyclic nucleotides and their effects on signal transduction. At least seven families of mammalian PDEs have been identified based on substrate specificity and affinity, sensitivity to cofactors, and sensitivity to inhibitory drugs (Beavo, J.A. (1995) Physiological Reviews 75: 725-48). Most of these families contain distinct genes; many of which are expressed in different tissues as alternative splice variants. Within families, there are multiple isozymes and multiple splice variants of those isozymes. The existence of multiple PDE families, isozymes, and splice variants presents an opportunity for regulation of cyclic nucleotide levels and functions.

Type 1 PDEs (PDE1s) are Ca^{2+}/calmodulin dependent, are reported to contain three

different genes, each of which appears to have at least two different splice variants, and have been found in the lung, heart, and brain. Some of the calmodulin-dependent PDEs are regulated *in vitro* by phosphorylation/dephosphorylation. Phosphorylation of PDE1 decreases the affinity of the enzyme for calmodulin, decreases PDE activity, and increases steady state levels of cAMP. PDE2s are cGMP stimulated PDEs that are localized in the brain and that are thought to mediate the effects of cAMP on catecholamine secretion. PDE3s are one of the major families of PDEs present in vascular smooth muscle. PDE3s are inhibited by cGMP, have high specificity for cAMP as a substrate, and play a role in cardiac function. One isozyme of PDE3 is regulated by one or more insulin-dependent kinases. PDE4s are the predominant isoenzymes in most inflammatory cells, some PDE4s are activated by cAMP-dependent phosphorylation. PDE5s are thought to be cGMP specific, but they may also affect cAMP function. High levels of PDE5s are found in most smooth muscle preparations, in platelets, and in the kidney. PDE6s play a role in vision and are regulated by light and cGMP. The PDE7 class has only one known member. PDE7 is cAMP specific and is most closely related to PDE4, although it is not inhibited by rolipram, a specific inhibitor of PDE4. A complete listing of PDE families 1, 2, 3, 4, 5, 6, and 7; their localization; and their physiological roles is given in Beavo, supra.

PDEs are composed of a catalytic domain of ~270 amino acid, an N-terminal regulatory domain responsible for binding cofactors, and, in some cases, a C-terminal domain of unknown function. A conserved motif, HDXXHXGXXN (SEQ ID NO:17), has been identified in the catalytic domain of all PDEs. PDE families display approximately 30% amino acid identity within this catalytic domain; while within the same family, isozymes typically display about 85-95% identity in this region (e.g. PDE4A vs PDE4B). Furthermore, within a family there is extensive similarity (>60%) outside the catalytic domain; while across families, there is little or no sequence similarity.

Many functions of immune and inflammatory responses are inhibited by agents that increase intracellular levels of cAMP (Verghese, M.W. et al. (1995) Mol Pharmacol 47:1164-1171). A variety of diseases have been attributed to increased PDE activity and associated with decreased levels of cyclic nucleotides. A form of diabetes insipidus in the mouse has been associated with increased PDE4 activity, and an increase in low-K_m cAMP PDE activity

has been reported in leukocytes of atopic patients. Defects in PDEs have also been associated with retinal disease. Retinal degeneration in the rd mouse, autosomal recessive retinitis pigmentosa in humans, and rod/cone dysplasia 1 in Irish Setter dogs have been attributed to mutations in the PDE6B gene. PDE3 has been associated with cardiac disease.

Many inhibitors of PDEs have been identified and have undergone clinical evaluation. PDE3 inhibitors are being developed as antithrombotic agents, antihypertensive agents, and cardiotonic agents useful in the treatment of congestive heart failure. Rolipram, a PDE4 inhibitor, has been used in the treatment of depression, and other inhibitors of PDE4 are undergoing evaluation as anti-inflammatory agents. Rolipram has also been shown to inhibit lipopolysaccharide (LPS) induced TNF-alpha, which has been shown to enhance HIV-1 replication *in vitro*. Therefore, rolipram may inhibit HIV-1 replication (Angel, J.B. et al. (1995) AIDS 9:1137-44). Additionally, rolipram, based on its ability to suppress the production of cytokines such as TNF alpha and beta and interferon gamma, has been shown to be effective in the treatment of encephalomyelitis. Rolipram may also be effective in treating tardive dyskinesia and was effective in treating multiple sclerosis in an experimental animal model (Sommer, N. et al., (1995) Nat .Med. 1:244-248; Sasaki, H. et al. (1995) Eur. J. Pharmacol 282:71-76).

Theophylline is a nonspecific PDE inhibitor used in the treatment of bronchial asthma and other respiratory diseases. Theophylline is believed to act on airway smooth muscle function and in an anti-inflammatory or immunomodulatory capacity in the treatment of respiratory diseases (Banner, K.H.. and Page, C.P. (1995) Eur. Respir. J. 8:996-1000). Pentoxifylline is another nonspecific PDE inhibitor used in the treatment of intermittent claudication and diabetes-induced peripheral vascular disease. Pentoxifylline is also known to block TNF-alpha production and may inhibit HIV-1 replication (Angel et al., supra).

PDEs have also been reported to effect cellular proliferation of a variety of cell types and have been implicated in various cancers. Bang et al. (1994; Proc Natl Acad Sci USA 91:5330-5334) reported that growth of prostate carcinoma cell lines DU 145 and LNCap, was inhibited by delivery of cAMP derivatives and phosphodiesterase inhibitors. Bang also observed a permanent conversion in phenotype from epithelial to neuronal morphology . Others have suggested that PDE inhibitors have the potential to regulate mesangial cell

proliferation and lymphocyte proliferation (Matousovic, K. et al. (1995) J. Clin. Invest .96:401-410; Joulain, C. et al. (1995) J. Lipid Mediat. Cell Signal. 11:63-79, respectively). Finally, Deonarain et al. describe a cancer treatment that involves intracellular delivery of phosphodiesterases to particular cellular compartments or tumors and that results in cell death (Deonarain, M.P. et al. (1994) Br. J.Cancer 70:786-94).

The discovery of new cyclic nucleotide phosphodiesterases and the polynucleotides encoding them satisfies a need in the art by providing new compositions which are useful in the diagnosis, prevention, and treatment of cancer and immune and neuronal disorders.

SUMMARY OF THE INVENTION

The invention features substantially purified polypeptides, cyclic nucleotide phosphodiesterases, referred to collectively as "PDE8" and individually as "PDE8A," "PDE8A(E)" (PDE8A extended), "PDE8B," and "PDE8B(E)" (PDE8B extended). In one aspect, the invention provides a substantially purified polypeptide, PDE8, comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, a fragment of SEQ ID NO:1, a fragment of SEQ ID NO:3, a fragment of SEQ ID NO:5, and a fragment of SEQ ID NO:7.

The invention further provides a substantially purified variant of PDE8 having at least 90% amino acid identity to the amino acid sequences of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7, a fragment of SEQ ID NO:1, a fragment of SEQ ID NO:3, a fragment of SEQ ID NO:5, or a fragment of SEQ ID NO:7. The invention also provides an isolated and purified polynucleotide sequence encoding the polypeptide comprising any of the amino acid sequences described above. The invention also includes an isolated and purified polynucleotide variant having at least 90% polynucleotide identity to the polynucleotide sequence encoding the polypeptide comprising these amino acid sequences.

Additionally, the invention provides a composition comprising a polynucleotide sequence encoding the polypeptide comprising the amino acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, a fragment of SEQ ID NO:1, a fragment of SEQ ID NO:3, a fragment of SEQ ID NO:5, or a fragment of SEQ ID NO:7. The invention further provides an isolated and purified polynucleotide sequence which hybridizes under

stringent conditions to one of these polynucleotide sequences, as well as an isolated and purified polynucleotide sequence which is complementary to one of these polynucleotide sequences.

The invention also provides an isolated and purified polynucleotide sequence comprising a polynucleotide sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, a fragment of SEQ ID NO:2, a fragment of SEQ ID NO:4, a fragment of SEQ ID NO:6, and a fragment of SEQ ID NO:8. The invention further provides an isolated and purified polynucleotide variant having at least 90% polynucleotide identity to one of these polynucleotide sequences, as well as an isolated and purified polynucleotide sequence which is complementary to one of these polynucleotide sequences.

The invention further provides an expression vector containing at least a fragment of any of the claimed polynucleotide sequences. In another aspect, the expression vector containing the polynucleotide sequence is contained within a host cell.

The invention also provides a method for producing a polypeptide comprising the amino acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, a fragment of SEQ ID NO:1, a fragment of SEQ ID NO:3, a fragment of SEQ ID NO:5, or a fragment of SEQ ID NO:7, the method comprising the steps of: (a) culturing the host cell containing an expression vector containing at least a fragment of a polynucleotide sequence encoding PDE8 under conditions suitable for the expression of the polypeptide; and (b) recovering the polypeptide from the host cell culture.

The invention also provides a pharmaceutical composition comprising a substantially purified PDE8 having the amino acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, a fragment of SEQ ID NO:1, a fragment of SEQ ID NO:3, a fragment of SEQ ID NO:5, or a fragment of SEQ ID NO:7 in conjunction with a suitable pharmaceutical carrier.

The invention further includes a purified antibody which binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, a fragment of SEQ ID NO:1, a fragment of SEQ ID NO:3, a fragment of SEQ ID NO:5, or a fragment of SEQ ID NO:7, as well as a purified agonist and a purified antagonist

to the polypeptide.

The invention also provides a method for treating or preventing a cancer, the method comprising administering to a subject in need of such treatment an effective amount of an antagonist of PDE8.

The invention also provides a method for treating or preventing an immune disorder, the method comprising administering to a subject in need of such treatment an effective amount of an antagonist of PDE8.

The invention also provides a method for treating or preventing a neuronal disorder, the method comprising administering to a subject in need of such treatment an effective amount of an antagonist of PDE8.

The invention also provides a method for detecting a polynucleotide encoding PDE8 in a biological sample containing nucleic acids, the method comprising the steps of: (a) hybridizing the complement of the polynucleotide sequence which encodes the polypeptide comprising SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, a fragment of SEQ ID NO:1, a fragment of SEQ ID NO:3, a fragment of SEQ ID NO:5, or a fragment of SEQ ID NO:7 to at least one of the nucleic acids of the biological sample, thereby forming a hybridization complex; and (b) detecting the hybridization complex, wherein the presence of the hybridization complex correlates with the presence of a polynucleotide encoding PDE8 in the biological sample. In one aspect, the nucleic acids of the biological sample are amplified by the polymerase chain reaction prior to the hybridizing step.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A, 1B, 1C, 1D, 1E, and 1F show the amino acid sequence (SEQ ID NO:1) and nucleic acid sequence (SEQ ID NO:2) of PDE8A.

Figure 2 shows the amino acid sequence (SEQ ID NO:3) and nucleic acid sequence (SEQ ID NO:4) of PDE8B.

Figures 3A, 3B, 3C, 3D, 3E, 3F, 3G, 3H, and 3I show the amino acid sequence (SEQ ID NO:5) and nucleic acid sequence (SEQ ID NO:6) of PDE8A(E).

Figures 4A, 4B, 4C, 4D, 4E, 4F, and 4G show the amino acid sequence (SEQ ID NO:7) and nucleic acid sequence (SEQ ID NO:8) of PDE8B(E).

The alignments were produced using MACDNASIS PRO software (Hitachi Software Engineering Co. Ltd. San Bruno, CA).

Figures 5A, 5B, 5C, 5D, 5E, and 5F show the amino acid sequence alignments among PDE8A (SEQ ID NO:1), PDE8B (SEQ ID NO:3), PDE8A(E) (SEQ ID NO:5) , PDE8B(E) (SEQ ID NO:7), and rat PDE4A(GI 1705952; SEQ ID NO:9)), produced using the multisequence alignment program of LASERGENE software.

Figure 6 shows the double-reciprocal, Lineweaver-Burke plot for the activity of PDE8A(E) using cAMP as a substrate; the positive X axis reflects the recriprocal of the substrate (cAMP) concentration (1/S), and the positive Y axis reflects the recriprocal of the reaction velocity (1/V). Lineweaver-Burke analysis was performed according to Segal, I.H. (Enzyme Kinetics (1995) pp. 214-245, John Wiley and Sons, New York, N. Y.)

Figure 7 shows the dependence of PDE8A(E) activity on divalent cation concentration; the positive X axis reflects cation concentration (mM), and the positive Y axis reflects the percent hydrolysis of cAMP. Divalent cations tested were calcium chloride ($CaCL_2$; circles), magnesium chloride ($MgCl_2$; squares), and manganese chloride ($MnCl_2$; diamonds).

Figure 8 shows the effect of various PDE inhibitors on the activity of PDE8A(E); the positive X axis reflects the concentration of inhibitor (M), and the positive Y axis reflects the percent inhibition of the enzyme.

DESCRIPTION OF THE INVENTION

Before the present proteins, nucleotide sequences, and methods are described, it is understood that this invention is not limited to the particular methodology, protocols, cell lines, vectors, and reagents described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a host cell" includes a plurality of such host cells, reference

to the "antibody" is a reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the cell lines, vectors, and methodologies which are reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

DEFINITIONS

PDE8, as used herein, refers to the amino acid sequences of substantially purified PDE8 obtained from any species, particularly mammalian, including bovine, ovine, porcine, murine, equine, and preferably human, from any source whether natural, synthetic, semi-synthetic, or recombinant.

The term "agonist", as used herein, refers to a molecule which, when bound to PDE8, increases or prolongs the duration of the effect of PDE8. Agonists may include proteins, nucleic acids, carbohydrates, or any other molecules which bind to and modulate the effect of PDE8.

An "allele" or "allelic sequence", as used herein, is an alternative form of the gene encoding PDE8. Alleles may result from at least one mutation in the nucleic acid sequence and may result in altered mRNAs or polypeptides whose structure or function may or may not be altered. Any given natural or recombinant gene may have none, one, or many allelic forms. Common mutational changes which give rise to alleles are generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

"Altered" nucleic acid sequences encoding PDE8 as used herein include those with deletions, insertions, or substitutions of different nucleotides resulting in a polynucleotide that

encodes the same or a functionally equivalent PDE8. Included within this definition are polymorphisms which may or may not be readily detectable using a particular oligonucleotide probe of the polynucleotide encoding PDE8, and improper or unexpected hybridization to alleles, with a locus other than the normal chromosomal locus for the polynucleotide sequence encoding PDE8. The encoded protein may also be "altered" and contain deletions, insertions, or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent PDE8. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the biological or immunological activity of PDE8 is retained. For example, negatively charged amino acids may include aspartic acid and glutamic acid; positively charged amino acids may include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values may include leucine, isoleucine, and valine, glycine and alanine, asparagine and glutamine, serine and threonine, and phenylalanine and tyrosine.

"Amino acid sequence" as used herein refers to an oligopeptide, peptide, polypeptide, or protein sequence, and fragment thereof, and to naturally occurring or synthetic molecules. Fragments of PDE8 are preferably about 5 to about 15 amino acids in length and retain the biological activity or the immunological activity of PDE8. Where "amino acid sequence" is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, amino acid sequence, and like terms, are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

"Amplification" as used herein refers to the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction (PCR) technologies well known in the art (Dieffenbach, C.W. and G.S. Dveksler (1995) PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview, NY).

The term "antagonist" as used herein, refers to a molecule which, when bound to PDE8, decreases the amount or the duration of the effect of the biological or immunological activity of PDE8. Antagonists may include proteins, nucleic acids, carbohydrates, antibodies or any other molecules which decrease the effect of PDE8.

As used herein, the term "antibody" refers to intact molecules as well as fragments

thereof, such as Fa, $F(ab')_2$, and Fv, which are capable of binding the epitopic determinant. Antibodies that bind PDE8 polypeptides can be prepared using intact polypeptides or fragments containing small peptides of interest as the immunizing antigen. The polypeptide or oligopeptide used to immunize an animal can be derived from the translation of RNA or synthesized chemically and can be conjugated to a carrier protein, if desired. Commonly used carriers that are chemically coupled to peptides include bovine serum albumin and thyroglobulin, keyhole limpet hemocyanin. The coupled peptide is then used to immunize the animal (e.g., a mouse, a rat, or a rabbit).

The term "antigenic determinant", as used herein, refers to that fragment of a molecule (i.e., an epitope) that makes contact with a particular antibody. When a protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies which bind specifically to a given region or three-dimensional structure on the protein; these regions or structures are referred to as antigenic determinants. An antigenic determinant may compete with the intact antigen (i.e., the immunogen used to elicit the immune response) for binding to an antibody.

The term "antisense", as used herein, refers to any composition containing nucleotide sequences which are complementary to a specific DNA or RNA sequence. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. Antisense molecules include peptide nucleic acids and may be produced by any method including synthesis or transcription. Once introduced into a cell, the complementary nucleotides combine with natural sequences produced by the cell to form duplexes and block either transcription or translation. The designation "negative" is sometimes used in reference to the antisense strand, and "positive" is sometimes used in reference to the sense strand.

The term "biologically active", as used herein, refers to a protein having structural, regulatory, or biochemical functions of a naturally occurring molecule. Likewise, "immunologically active" refers to the capability of the natural, recombinant, or synthetic PDE8, or any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells and to bind with specific antibodies.

The terms "complementary" or "complementarity", as used herein, refer to the natural

binding of polynucleotides under permissive salt and temperature conditions by base-pairing. For example, the sequence "A-G-T" binds to the complementary sequence "T-C-A". Complementarity between two single-stranded molecules may be "partial", in which only some of the nucleic acids bind, or it may be complete when total complementarity exists between the single stranded molecules. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, which depend upon binding between nucleic acids strands and in the design and use of PNA molecules.

A "composition comprising a given polynucleotide sequence" as used herein refers broadly to any composition containing the given polynucleotide sequence. The composition may comprise a dry formulation or an aqueous solution. Compositions comprising polynucleotide sequences encoding PDE8 may be employed as hybridization probes. The probes may be stored in freeze-dried form and may be associated with a stabilizing agent such as a carbohydrate. In hybridizations, the probe may be deployed in an aqueous solution containing salts (e.g., NaCl), detergents (e.g., SDS) and other components (e.g., Denhardt's solution, dry milk, salmon sperm DNA, etc.).

"Consensus", as used herein, refers to a nucleic acid sequence which has been resequenced to resolve uncalled bases, has been extended using the XL-PCR kit (Perkin Elmer, Foster City, CA) in the 5' and/or the 3' direction and resequenced, or has been assembled from the overlapping sequences of more than one Incyte Clone using a computer program for fragment assembly (e.g., GELVIEW Fragment assembly system, GCG, Madison, WI). Some sequences have been both extended and assembled to produce the consensus sequence .

The term "correlates with expression of a polynucleotide", as used herein, indicates that the detection of the presence of ribonucleic acid that is similar to SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 by northern analysis is indicative of the presence of mRNA encoding PDE8 in a sample and thereby correlates with expression of the transcript from the polynucleotide encoding the protein.

A "deletion", as used herein, refers to a change in the amino acid or nucleotide sequence and results in the absence of one or more amino acid residues or nucleotides.

The term "derivative", as used herein, refers to the chemical modification of a nucleic acid encoding or complementary to PDE8 or the encoded PDE8. Such modifications include, for example, replacement of hydrogen by an alkyl, acyl, or amino group. A nucleic acid derivative encodes a polypeptide which retains the biological or immunological function of the natural molecule. A derivative polypeptide is one which is modified by glycosylation, pegylation, or any similar process which retains the biological or immunological function of the polypeptide from which it was derived.

The term "homology", as used herein, refers to a degree of complementarity. There may be partial homology or complete homology (i.e., identity). A partially complementary sequence that at least partially inhibits an identical sequence from hybridizing to a target nucleic acid is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or hybridization probe will compete for and inhibit the binding of a completely homologous sequence to the target sequence under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (i.e., selective) interaction. The absence of non-specific binding may be tested by the use of a second target sequence which lacks even a partial degree of complementarity (e.g., less than about 30% identity). In the absence of non-specific binding, the probe will not hybridize to the second non-complementary target sequence.

Human artificial chromosomes (HACs) are linear microchromosomes which may contain DNA sequences of 10K to 10M in size and contain all of the elements required for stable mitotic chromosome segregation and maintenance (Harrington, J.J. et al. (1997) Nat Genet. 15:345-355).

The term "humanized antibody", as used herein, refers to antibody molecules in which amino acids have been replaced in the non-antigen binding regions in order to more closely resemble a human antibody, while still retaining the original binding ability.

The term "hybridization", as used herein, refers to any process by which a strand of

nucleic acid binds with a complementary strand through base pairing.

The term "hybridization complex", as used herein, refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bonds between complementary G and C bases and between complementary A and T bases; these hydrogen bonds may be further stabilized by base stacking interactions. The two complementary nucleic acid sequences hydrogen bond in an antiparallel configuration. A hybridization complex may be formed in solution (e.g., C_0t or R_0t analysis) or between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized on a solid support (e.g., paper, membranes, filters, chips, pins or glass slides, or any other appropriate substrate to which cells or their nucleic acids have been fixed).

An "insertion" or "addition", as used herein, refers to a change in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid residues or nucleotides, respectively, as compared to the naturally occurring molecule.

"Microarray" refers to an array of distinct polynucleotides or oligonucleotides arranged on a substrate, such as paper, nylon or other type of membrane, filter, chip, glass slide, or any other suitable solid support.

The term "modulate", as used herein, refers to a change in the activity of PDE8. For example, modulation may cause an increase or a decrease in protein activity, binding characteristics, or any other biological, functional or immunological properties of PDE8.

"Nucleic acid sequence" as used herein refers to an oligonucleotide, nucleotide, or polynucleotide, and fragments thereof, and to DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand. "Fragments" are those nucleic acid sequences which are greater than 60 nucleotides in length, and most preferably includes fragments that are at least 100 nucleotides or at least 1000 nucleotides, and at least 10,000 nucleotides in length.

The term "oligonucleotide" refers to a nucleic acid sequence of at least about 6 nucleotides to about 60 nucleotides, preferably about 15 to 30 nucleotides, and more preferably about 20 to 25 nucleotides, which can be used in PCR amplification or a hybridization assay, or a microarray. As used herein, oligonucleotide is substantially equivalent to the terms "amplimers","primers", "oligomers", and "probes", as commonly

defined in the art.

"Peptide nucleic acid", PNA as used herein, refers to an antisense molecule or anti-gene agent which comprises an oligonucleotide of at least five nucleotides in length linked to a peptide backbone of amino acid residues which ends in lysine. The terminal lysine confers solubility to the composition. PNAs may be pegylated to extend their lifespan in the cell where they preferentially bind complementary single stranded DNA and RNA and stop transcript elongation (Nielsen, P.E. et al. (1993) Anticancer Drug Des. 8:53-63).

The term "portion", as used herein, with regard to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from five amino acid residues to the entire amino acid sequence minus one amino acid. Thus, for example, a protein "comprising at least a portion of the amino acid sequence of SEQ ID NO:1" encompasses the full-length PDE8A and fragments thereof.

The term "sample", as used herein, is used in its broadest sense. A biological sample suspected of containing nucleic acid encoding PDE8, or fragments thereof, or PDE8 itself may comprise a bodily fluid, extract from a cell, chromosome, organelle, or membrane isolated from a cell, a cell, genomic DNA, RNA, or cDNA (in solution or bound to a solid support, a tissue, a tissue print, and the like).

The terms "specific binding" or "specifically binding", as used herein, refers to that interaction between a protein or peptide and an agonist, an antibody and an antagonist. The interaction is dependent upon the presence of a particular structure (i.e., the antigenic determinant or epitope) of the protein recognized by the binding molecule. For example, if an antibody is specific for epitope "A", the presence of a protein containing epitope A (or free, unlabeled A) in a reaction containing labeled "A" and the antibody will reduce the amount of labeled A bound to the antibody.

As used herein, the term "stringent conditions" refers to conditions which permit hybridization between polynucleotide sequences and the claimed polynucleotide sequences. Suitably stringent conditions can be defined by, for example, the concentrations of salt and/or formamide in the prehybridization and hybridization solutions, or by the hybridization temperature, and are well known in the art. In particular, stringency can be increased by reducing the concentration of salt, increasing the concentration of formamide, or raising the

hybridization temperature.

For example, hybridization under high stringency conditions could occur in about 50% formamide at about 37°C to 42°C. Hybridization could occur under reduced stringency conditions in about 35% to 25% formamide at about 30°C to 35°C. In particular, hybridization could occur under high stringency conditions at 42°C in 50% formamide, 5X SSPE, 0.3% SDS, and 200 μg/ml sheared and denatured salmon sperm DNA. Hybridization could occur under reduced stringency conditions as described above, but in 35% formamide or/and at a reduced temperature of 35°C. The temperature range corresponding to a particular level of stringency can be further narrowed by calculating the purine to pyrimidine ratio of the nucleic acid of interest and adjusting the temperature accordingly. Variations on the above ranges and conditions are well known in the art.

The term "substantially purified", as used herein, refers to nucleic or amino acid sequences that are removed from their natural environment, isolated or separated, and are at least 60% free, preferably 75% free, and most preferably 90% free from other components with which they are naturally associated.

A "substitution", as used herein, refers to the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively.

"Transformation", as defined herein, describes a process by which exogenous DNA enters and changes a recipient cell. It may occur under natural or artificial conditions using various methods well known in the art. Transformation may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method is selected based on the type of host cell being transformed and may include, but is not limited to, viral infection, electroporation, heat shock, lipofection, and particle bombardment. Such "transformed" cells include stably transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome. They also include cells which transiently express the inserted DNA or RNA for limited periods of time.

A "variant" of PDE8, as used herein, refers to an amino acid sequence that is altered by one or more amino acids. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, e.g., replacement of

leucine with isoleucine. More rarely, a variant may have "nonconservative" changes, e.g., replacement of a glycine with a tryptophan. Analogous minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing biological or immunological activity may be found using computer programs well known in the art, for example, SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, and SEQ ID NO:7 software.

THE INVENTION

The invention is based on the discovery and extension of nucleotide sequences for a new family of human cyclic nucleotide phosphodiesterases, PDE8, the polynucleotides encoding PDE8, and the use of these compositions for the diagnosis, prevention, or treatment of cancer and immune and neuronal disorders.

Nucleic acids encoding the PDE8A of the present invention were first identified in Incyte Clone 156196 from the promonocyte cell line cDNA library (THP1PLB02) using a computer search for amino acid sequence alignments. A consensus sequence, SEQ ID NO:2, was derived from extension of the nucleic acid sequence of this clone.

In one embodiment, the invention encompasses a polypeptide comprising the amino acid sequence of SEQ ID NO:1, as shown in Figs. 1A, 1B, 1C, 1D, 1E, and 1F. PDE8A is 449 amino acids in length and has a consensus signature sequence for cyclic nucleotide PDEs at H_{216}DVDHPGRTN of SEQ ID NO:1. This sequence is a part of one of two potential divalent cation binding sites conserved in PDEs having the general structure of HXXXH(X_{6-24})E (SEQ ID NO:18). The first of these sites is found in the sequence H_{176}---H_{180}---D_{205}, and has D_{205} as a conservative amino acid substitution for E. This substitution is found in at least one other PDE, PDE7. The second of these sites is found in the sequence H_{216}---H_{220}---E_{246}. As shown in Figs. 5A, 5B, 5C, 5D, 5E, and 5F, PDE8A has chemical and structural homology with PDE8B (SEQ ID NO:3), PDE8A(E) (SEQ ID NO:5), PDE8B(E) (SEQ ID NO:7), and rat PDE4A (GI 1705952; SEQ ID NO:9). In particular, PDE8A shares 70% identity with PDE8B in the C-terminal portion of PDE8A beginning at residue I_{304}. PDE8A shares 99% identity with PDE8A(E) beginning at residue M_{265} in PDE8A(E). PDE8A shares 78% identity with PDE8B(E), and 29% identity with rat PDE4. The ~ 270 amino acid

catalytic domain found in all PDEs extends approximately between residues L_{151} and W_{433} for PDE8A, and is 33% identical to rat PDE4A in this region. All five proteins share the two divalent cation binding sites and the consensus signature sequence, HDXXHXGXXN (SEQ ID NO:17). Electronic northern analysis using the LIFESEQ database (Incyte Corporation, Palo Alto, CA) shows the expression of PDE8A in various libraries, at least 20% of which are immortalized or cancerous and at least 80% of which involve the immune response.

Nucleic acids encoding the PDE8B of the present invention were first identified in Incyte Clone 464655 from the atrial tissue cDNA library (LATRNOT01) using a computer search for amino acid sequence alignments. A consensus sequence, SEQ ID NO:4, was derived from extension of the nucleic acid sequence of this clone.

In another embodiment, the invention encompasses a polypeptide comprising the amino acid sequence of SEQ ID NO:3, as shown in Fig. 2. PDE8B is 81 amino acids in length. As shown in Figs.5A, 5B, 5C, 5D, 5E, and 5F, PDE8B shares 100% identity with the C-terminal portion of PDE8B(E) beginning at residue I_{568} of the latter protein. PDE8B shares 27% identity with rat PDE4A. Electronic northern analysis shows the expression of this sequence in various libraries, at least 43% of which involve cancer, and at least 43% of which involve the brain and neural tissue.

Nucleic acids encoding the PDE8A(E) of the present invention were first identified in Incyte Clone 156196 from the promonocyte cell line cDNA library (THP1PLB02) using a computer search for amino acid sequence alignments. SEQ ID NO:6 was derived from further extension of Incyte Clone 156196.

In another embodiment, the invention encompasses a polypeptide comprising the amino acid sequence of SEQ ID NO:5, as shown in Figs. 3A, 3B, 3C, 3D, 3E, 3F, 3H, and 3I. PDE8A(E) is 713 amino acids in length and has as consensus signature sequence for cyclic nucleotide PDEs beginning at H_{480}. The two divalent cation binding sites begin at residues H_{440} and H_{480}. As shown in Figs.5A, 5B, 5C, 5D, 5E, and 5F, PDE8A(E) shares chemical and structural homology with the other PDE8 proteins and with rat PDE4A. In particular, the C-terminal portion of PDE8A(E) is identical (100%) with PDE8A beginning at residue I_{568}. PDE8A(E) shares 22% overall identity with rat PDE4 and 35% identity in the catalytic domain of PDE4.

A 1.6 kb region of PDE8A(E) encoding the C-terminal 545 amino acids was cloned into the baculovirus transfer vector pFASTBAC, expressed in sf9 cells, and a cell lysate prepared from these cells for enzyme assays. Fig. 6 shows the kinetics of enzyme activity of recombinant, purified PDE8A(E) with cAMP as a substrate. PDE8A(E) has a very high affinity for cAMP with a Km of 55 nM, and a very low affinity for cGMP (Km = 124 mM, data not shown). Fig. 7 shows the dependence of PDE8A(E) on divalent cations for maximal activity with a preference for Mn^{++}or Mg^{++} over Ca^{++}. The effects of various known PDE inhibitors on the activity of PDE8A(E) are shown in Fig.8. PDE8A(E) was not inhibited by up to 100 mM of rolipram, SKF94120 (inhibitor of PDE3), zaprinast (inhibitor of PDE5), vinpocetine (inhibitor of PDE1), of IBMX (non-specific PDE inhibitor). PDE8A(E) was inhibited by dipyridamole (inhibitor of PDE5) with an IC_{50} of 9 μM. Membrane-based northern analysis shows the expression of this sequence in various tissues, with the most significant expression in testis, ovary, small intestine, and colon.

Nucleic acids encoding the PDE8B(E) of the present invention were first identified in Incyte Clone 464655 from the atrial tissue cDNA library (LATRNOT01) using a computer search for amino acid sequence alignments. SEQ ID NO:8 was derived from extension and assembly of Incyte Clones 464655 (LATRNOT01) and 112633 (PITUNOT01).

In another embodiment, the invention encompasses a polypeptide comprising the amino acid sequence of SEQ ID NO:7, as shown in Figs. 4A, 4B, 4C, 4D, 4E, 4F, and 4G. PDE8B(E) is 718 amino acids in length and has a consensus cyclic nucleotide PDE signature sequence beginning at H_{488}. The two divalent cation binding sites begin at residues H_{448} and H_{488}. As shown in Figs.5A, 5B, 5C, 5D, 5E, and 5F, PDE8B(E) has chemical and structural homology with the other PDE8 proteins and with rat PDE4A. In particular, the C-terminal portion of PDE8B(E) is identical (100%) with PDE8B between residues I_{576} and D_{656} of PDE8B(E). PDE8B(E) shares 71% identity with PDE8A(E) and 22% identity with rat PDE4A.

The degree of similarity exhibited among the four PDE8 proteins (70% to 100%) is consistent with that shown between isozymes within the same family, while the degree of similarity between the four PDE8 proteins and PDE4 (22% to 29%) is consistent with that shown between isozymes of different families. PDE8A(E) is further distinguished from other

known families by its specificity for cAMP and pattern of inhibition by known PDE inhibitors. PDE8A, PDE8A(E), PDE8B, and PDE8B(E) therefore appear to constitute a new family of cyclic nucleotide phosphodiesterases designated PDE8.

The invention also encompasses PDE8 variants. A preferred PDE8 variant is one having at least 80%, and more preferably at least 90%, amino acid sequence identity to the PDE8 amino acid sequence claimed herein and which retains at least one biological, immunological or other functional characteristic or activity of PDE8. A most preferred PDE8 variant is one having at least 95% amino acid sequence identity.

The invention also encompasses polynucleotide sequences which encode PDE8. Accordingly, any nucleic acid sequence which encodes the amino acid sequence of PDE8 can be used to produce recombinant molecules which express PDE8. In a particular embodiment, the invention encompasses the polynucleotide comprising the nucleic acid sequence of SEQ ID NO:2 as shown in Figs. 1A, 1B, 1C, 1D, 1E, and 1F. In another embodiment, the invention encompasses the polynucleotide comprising the nucleic acid sequence of SEQ ID NO:4 as shown in Fig.2. In another embodiment, the invention encompasses the polynucleotide comprising the nucleic acid sequence of SEQ ID NO:6 as shown in Figs.3A, 3B, 3C, 3D, 3E, 3F, 3G, 3H, and 3I. In still another embodiment, the invention encompasses the polynucleotide comprising the nucleic acid sequence of SEQ ID NO:8 as shown in Figs. 4A, 4B, 4C, 4D, 4E, 4F, and 4G.

The invention also encompasses a variant of a polynucleotide sequence encoding PDE8. In particular, such a variant polynucleotide sequence will have at least about 80%, more preferably at least about 90%, and most preferably at least about 95% polynucleotide sequence identity to the polynucleotide sequence encoding PDE8. A particular aspect of the invention encompasses a variant of SEQ ID NO:2 which has at least about 80%, more preferably at least about 90%, and most preferably at least about 95% polynucleotide sequence identity to SEQ ID NO:2. The invention further encompasses a polynucleotide variant of SEQ ID NO:4 which has at least at least about 80%, more preferably at least about 90%, and most preferably at least about 95% polynucleotide sequence identity to SEQ ID NO:4. Another aspect of the invention encompasses a variant of SEQ ID NO:6 which has at least about 80%, more preferably at least about 90%, and most preferably at least about 95%

polynucleotide sequence identity to SEQ ID NO:6. The invention also encompasses a polynucleotide variant of SEQ ID NO:8 having at least about 80%, more preferably at least about 90%, and most preferably at least about 95% polynucleotide sequence identity to SEQ ID NO:8.

It will be appreciated by those skilled in the art that as a result of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding PDE8, some bearing minimal homology to the nucleotide sequences of any known and naturally occurring gene, may be produced. Thus, the invention contemplates each and every possible variation of nucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the nucleotide sequence of naturally occurring PDE8, and all such variations are to be considered as being specifically disclosed.

Although nucleotide sequences which encode PDE8 and its variants are preferably capable of hybridizing to the nucleotide sequence of the naturally occurring PDE8 under appropriately selected conditions of stringency, it may be advantageous to produce nucleotide sequences encoding PDE8 or its derivatives possessing a substantially different codon usage. Codons may be selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic host in accordance with the frequency with which particular codons are utilized by the host. Other reasons for substantially altering the nucleotide sequence encoding PDE8 and its derivatives without altering the encoded amino acid sequences include the production of RNA transcripts having more desirable properties, such as a greater half-life, than transcripts produced from the naturally occurring sequence.

The invention also encompasses production of DNA sequences, or fragments thereof, which encode PDE8 and its derivatives, entirely by synthetic chemistry. After production, the synthetic sequence may be inserted into any of the many available expression vectors and cell systems using reagents that are well known in the art. Moreover, synthetic chemistry may be used to introduce mutations into a sequence encoding PDE8 or any fragment thereof.

Also encompassed by the invention are polynucleotide sequences that are capable of hybridizing to the claimed nucleotide sequences, and in particular, those shown in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8, under various conditions of

stringency as taught in Wahl, G.M. and S.L. Berger (1987; Methods Enzymol. 152:399-407) and Kimmel, A.R. (1987; Methods Enzymol. 152:507-511).

Methods for DNA sequencing which are well known and generally available in the art and may be used to practice any of the embodiments of the invention. The methods may employ such enzymes as the Klenow fragment of DNA polymerase I, SEQUENASE, Taq polymerase, thermostable T7 polymerase (Amersham, Pharmacia), or combinations of polymerases and proofreading exonucleases such as those found in the ELONGASE amplification system marketed by Life Technologies (Gaithersburg, MD). Preferably, the process is automated with machines such as the MICROLAB 2200 liquid transfer system (Hamilton, Reno, NV), PTC200 thermocycler DNA ENGINE (MJ Research, Watertown, MA) and the ABI CATALYST and 373 and 377 DNA Sequencers (PE Biosystems).

The nucleic acid sequences encoding PDE8 may be extended utilizing a partial nucleotide sequence and employing various methods known in the art to detect upstream sequences such as promoters and regulatory elements. For example, one method which may be employed, "restriction-site" PCR, uses universal primers to retrieve unknown sequence adjacent to a known locus (Sarkar, G. (1993) PCR Methods Applic. 2:318-322). In particular, genomic DNA is first amplified in the presence of primer to a linker sequence and a primer specific to the known region. The amplified sequences are then subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse transcriptase.

Inverse PCR may also be used to amplify or extend sequences using divergent primers based on a known region (Triglia, T. et al. (1988) Nucleic Acids Res. 16:8186). The primers may be designed using commercially available software such as OLIGO 4.06 primer analysis software (Inc., Plymouth, MN), or another appropriate program, to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68°-72° C. The method uses several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is then circularized by intramolecular ligation and used as a PCR template.

Another method which may be used is capture PCR which involves PCR

amplification of DNA fragments adjacent to a known sequence in human and yeast artificial chromosome DNA (Lagerstrom, M. et al. (1991) PCR Methods Applic. 1:111-119). In this method, multiple restriction enzyme digestions and ligations may also be used to place an engineered double-stranded sequence into an unknown fragment of the DNA molecule before performing PCR.

Another method which may be used to retrieve unknown sequences is that of Parker, J.D. et al. (1991; Nucleic Acids Res. 19:3055-3060). Additionally, one may use PCR, nested primers, and PROMOTERFINDER libraries (Clontech, Palo Alto, CA) to walk genomic DNA. This process avoids the need to screen libraries and is useful in finding intron/exon junctions. When screening for full-length cDNAs, it is preferable to use libraries that have been size-selected to include larger cDNAs. Also, random-primed libraries are preferable, in that they will contain more sequences which contain the 5' regions of genes. Use of a randomly primed library may be especially preferable for situations in which an oligo d(T) library does not yield a full-length cDNA. Genomic libraries may be useful for extension of sequence into 5' non-transcribed regulatory regions.

Capillary electrophoresis systems which are commercially available may be used to analyze the size or confirm the nucleotide sequence of sequencing or PCR products. In particular, capillary sequencing may employ flowable polymers for electrophoretic separation, four different fluorescent dyes (one for each nucleotide) which are laser activated, and detection of the emitted wavelengths by a charge coupled device camera. Output/light intensity may be converted to electrical signal using appropriate software (e.g. GENOTYPER and NAVIGATOR, PE Biosystems) and the entire process from loading of samples to computer analysis and electronic data display may be computer controlled. Capillary electrophoresis is especially preferable for the sequencing of small pieces of DNA which might be present in limited amounts in a particular sample.

In another embodiment of the invention, polynucleotide sequences or fragments thereof which encode PDE8 may be used in recombinant DNA molecules to direct expression of PDE8, fragments or functional equivalents thereof, in appropriate host cells. Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence may be produced, and these sequences

may be used to clone and express PDE8.

As will be understood by those of skill in the art, it may be advantageous to produce PDE8-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of protein expression or to produce an RNA transcript having desirable properties, such as a half-life which is longer than that of a transcript generated from the naturally occurring sequence.

The nucleotide sequences of the present invention can be engineered using methods generally known in the art in order to alter PDE8 encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the gene product. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides may be used to engineer the nucleotide sequences. For example, site-directed mutagenesis may be used to insert new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, introduce mutations, and so forth.

In another embodiment of the invention, natural, modified, or recombinant nucleic acid sequences encoding PDE8 may be ligated to a heterologous sequence to encode a fusion protein. For example, to screen peptide libraries for inhibitors of PDE8 activity, it may be useful to encode a chimeric PDE8 protein that can be recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between the PDE8 encoding sequence and the heterologous protein sequence, so that PDE8 may be cleaved and purified away from the heterologous moiety.

In another embodiment, sequences encoding PDE8 may be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers, M.H. et al. (1980) Nucl. Acids Symp. Ser. 7:215-223, Horn, T. et al. (1980) Nucl. Acids Symp. Ser. 7:225-232). Alternatively, the protein itself may be produced using chemical methods to synthesize the amino acid sequence of PDE8, or a fragment thereof. For example, peptide synthesis can be performed using various solid-phase techniques (Roberge, J.Y. et al. (1995) Science 269:202-204) and automated synthesis may be achieved, for example, using an ABI 431A peptide synthesizer (PE Biosystems).

The newly synthesized peptide may be substantially purified by preparative high performance liquid chromatography (e.g., Creighton, T. (1983) Proteins, Structures and Molecular Principles, WH Freeman and Co., New York, NY). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; Creighton, supra). Additionally, the amino acid sequence of PDE8, or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins, or any part thereof, to produce a variant polypeptide.

In order to express a biologically active PDE8, the nucleotide sequences encoding PDE8 or functional equivalents, may be inserted into appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence.

Methods which are well known to those skilled in the art may be used to construct expression vectors containing sequences encoding PDE8 and appropriate transcriptional and translational control elements. These methods include in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. Such techniques are described in Sambrook, J. et al. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview, NY, and Ausubel, F.M. et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY.

A variety of expression vector/host systems may be utilized to contain and express sequences encoding PDE8. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (e.g., baculovirus); plant cell systems transformed with virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids); or animal cell systems. The invention is not limited by the host cell employed.

The "control elements" or "regulatory sequences" are those non-translated regions of the vector--enhancers, promoters, 5' and 3' untranslated regions--which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their

strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the BLUESCRIPT phagemid (Stratagene, LaJolla, CA) or PSPORT1 plasmid (Life Technologies) and the like may be used. The baculovirus polyhedrin promoter may be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (e.g., heat shock, RUBISCO; and storage protein genes) or from plant viruses (e.g., viral promoters or leader sequences) may be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell line that contains multiple copies of the sequence encoding PDE8, vectors based on SV40 or EBV may be used with an appropriate selectable marker.

In bacterial systems, a number of expression vectors may be selected depending upon the use intended for PDE8. For example, when large quantities of PDE8 are needed for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified may be used. Such vectors include, but are not limited to, the multifunctional E. coli cloning and expression vectors such as BLUESCRIPT (Stratagene), in which the sequence encoding PDE8 may be ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent 7 residues of ß-galactosidase so that a hybrid protein is produced; pIN vectors (Van Heeke, G. and S.M. Schuster (1989) J. Biol. Chem. 264:5503-5509); and the like. PGEX vectors (Amersham Pharmacia Biotech) may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems may be designed to include heparin, thrombin, or factor XA protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

In the yeast, Saccharomyces cerevisiae, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase, and PGH may be used. For reviews, see Ausubel et al. (supra) and Grant et al. (1987) Methods Enzymol. 153:516-544.

In cases where plant expression vectors are used, the expression of sequences encoding PDE8 may be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV may be used alone or in combination with the omega leader sequence from TMV (Takamatsu, N. (1987) EMBO J. 6:307-311). Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters may be used (Coruzzi, G. et al. (1984) EMBO J. 3:1671-1680; Broglie, R. et al. (1984) Science 224:838-843; and Winter, J. et al. (1991) Results Probl. Cell Differ. 17:85-105). These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection. Such techniques are described in a number of generally available reviews (see, for example, Hobbs, S. or Murry, L.E. in McGraw Hill Yearbook of Science and Technology (1992) McGraw Hill, New York, NY; pp. 191-196).

An insect system may also be used to express PDE8. For example, a 1.6 kb region of PDE8A(E) encoding the C-terminal 545 amino acids was PCR-amplified and cloned into the baculovirus transfer vector pFASTBAC (Life Technologies, Inc., Gaithersburg, MD), which had been modified to include a 5' FLAG tag. Recombinant virus stocks were prepared according to the manufacturer's protocol. Sf9 cells were cultured in Sf900 II Sfm serum free media (Life Technologies Inc.) at 27°C. For expression, 1 x 10^8 Sf9 cells were infected at a multiplicity of infection of 5 in a final volume of 50 mls. Three days post-infection, the cells were harvested and enzyme-containing lysates were prepared. To monitor expression, 1 ml each of mock-infected and PDE8A(E)-infected cell lysate was electrophoresed in a polyacrylamide gel and either silver-stained by standard methods or transferred to nitrocellulose for western analysis with an anti-FLAG antibody (M2, Scientific Imaging System) at a concentration of 2 mg/ml.

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, sequences encoding PDE8 may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain a viable virus which is capable of expressing PDE8 in infected host cells (Logan, J. and Shenk, T. (1984) Proc. Natl. Acad. Sci. 81:3655-3659). In addition,

transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

Human artificial chromosomes (HACs) may also be employed to deliver larger fragments of DNA than can be contained and expressed in a plasmid. HACs of 6 to 10M are constructed and delivered via conventional delivery methods (liposomes, polycationic amino polymers, or vesicles) for therapeutic purposes.

Specific initiation signals may also be used to achieve more efficient translation of sequences encoding PDE8. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding PDE8, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a fragment thereof, is inserted, exogenous translational control signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used, such as those described in the literature (Scharf, D. et al. (1994) Results Probl. Cell Differ. 20:125-162).

In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the protein may also be used to facilitate correct insertion, folding and/or function. Different host cells which have specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, and WI38), are available from the American Type Culture Collection (ATCC; Bethesda, MD) and may be chosen to ensure the correct modification and processing of the foreign protein.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express PDE8 may be transformed using

expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be proliferated using tissue culture techniques appropriate to the cell type.

Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler, M. et al. (1977) Cell 11:223-32) and adenine phosphoribosyltransferase (Lowy, I. et al. (1980) Cell 22:817-23) genes which can be employed in tk^- or $aprt^-$ cells, respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection; for example, dhfr which confers resistance to methotrexate (Wigler, M. et al. (1980) Proc. Natl. Acad. Sci. 77:3567-70); npt, which confers resistance to the aminoglycosides, neomycin and G-418 (Colbere-Garapin, F. et al (1981) J. Mol. Biol. 150:1-14); and als or pat, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry, supra). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine (Hartman, S.C. and R.C. Mulligan (1988) Proc. Natl. Acad. Sci. 85:8047-51). Recently, the use of visible markers has gained popularity with such markers as anthocyanins, ß glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes, C.A. et al. (1995) Methods Mol. Biol. 55:121-131).

Although the presence/absence of marker gene expression suggests that the gene of interest is also present, its presence and expression may need to be confirmed. For example, if the sequence encoding PDE8 is inserted within a marker gene sequence, transformed cells containing sequences encoding PDE8 can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a sequence encoding

PDE8 under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the tandem gene as well.

Alternatively, host cells which contain the nucleic acid sequence encoding PDE8 and express PDE8 may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations and protein bioassay or immunoassay techniques which include membrane, solution, or chip based technologies for the detection and/or quantification of nucleic acid or protein.

The presence of polynucleotide sequences encoding PDE8 can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes or fragments or fragments of polynucleotides encoding PDE8. Nucleic acid amplification based assays involve the use of oligonucleotides or oligomers based on the sequences encoding PDE8 to detect transformants containing DNA or RNA encoding PDE8.

A variety of protocols for detecting and measuring the expression of PDE8, using either polyclonal or monoclonal antibodies specific for the protein are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on PDE8 is preferred, but a competitive binding assay may be employed. These and other assays are described, among other places, in Hampton, R. et al. (1990; Serological Methods, a Laboratory Manual, APS Press, St Paul, MN) and Maddox, D.E. et al. (1983; J. Exp. Med. 158:1211-1216).

A wide variety of labels and conjugation techniques are known by those skilled in the art and may be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides encoding PDE8 include oligolabeling, nick translation, end-labeling or PCR amplification using a labeled nucleotide. Alternatively, the sequences encoding PDE8, or any fragments thereof may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes in vitro by addition of an appropriate RNA polymerase such as T7, T3, or SP6 and labeled nucleotides. These procedures may be conducted using a variety of commercially available

kits (Amersham Pharmacia Biotech). Suitable reporter molecules or labels, which may be used for ease of detection, include radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Host cells transformed with nucleotide sequences encoding PDE8 may be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The protein produced by a transformed cell may be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode PDE8 may be designed to contain signal sequences which direct secretion of PDE8 through a prokaryotic or eukaryotic cell membrane. Other constructions may be used to join sequences encoding PDE8 to nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAG extension/affinity purification system (Immunex Corp., Seattle, WA). The inclusion of cleavable linker sequences such as those specific for Factor XA or enterokinase (Invitrogen, San Diego, CA) between the purification domain and PDE8 may be used to facilitate purification. One such expression vector provides for expression of a fusion protein containing PDE8 and a nucleic acid encoding 6 histidine residues preceding a thioredoxin or an enterokinase cleavage site. The histidine residues facilitate purification on IMIAC (immobilized metal ion affinity chromatography) as described in Porath, J. et al. (1992; Prot. Exp. Purif. 3: 263-281) while the enterokinase cleavage site provides a means for purifying PDE8 from the fusion protein. A discussion of vectors which contain fusion proteins is provided in Kroll, D.J. et al. (1993; DNA Cell Biol. 12:441-453).

In addition to recombinant production, fragments of PDE8 may be produced by direct peptide synthesis using solid-phase techniques (Merrifield J. (1963) J. Am. Chem. Soc. 85:2149-2154). Protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using an ABI 431A peptide

synthesizer (PE Biosystems). Various fragments of PDE8 may be chemically synthesized separately and combined using chemical methods to produce the full length molecule.

THERAPEUTICS

Chemical and structural homology exists between PDE8 and PDE4 from rat (GI 1705952). In addition, PDE8 is expressed in cancer, tissues associated with inflammation and the immune response, and brain. Therefore, PDE8 appears to play a role in cancer and immune and neuronal disorders. In particular, inhibitors of PDE have been shown to be effective in the treatment of these types of diseases and disorders.

Therefore, in one embodiment, an antagonist of PDE8 may be administered to a subject to prevent or treat a cancer. Such cancers may be, but are not limited to, adenocarcinoma, leukemia, lymphoma, melanoma, myeloma, sarcoma, and teratocarcinoma, and, in particular, cancers of the adrenal gland, bladder, bone, bone marrow, brain, breast, cervix, gall bladder, ganglia, gastrointestinal tract, heart, kidney, liver, lung, muscle, ovary, pancreas, parathyroid, penis, prostate, salivary glands, skin, spleen, testis, thymus, thyroid, and uterus. In one aspect, an antibody which specifically binds PDE8 may be used directly as an antagonist or indirectly as a targeting or delivery mechanism for bringing a pharmaceutical agent to cells or tissue which express PDE8.

In another embodiment, a vector expressing the complement of the polynucleotide encoding PDE8 may be administered to a subject to treat or prevent a cancer including, but not limited to, the types of cancer described above.

In another embodiment, an antagonist of PDE8 may be administered to a subject to prevent or treat an immune disorder. Such disorders may include, but are not limited to, AIDS, Addison's disease, adult respiratory distress syndrome, allergies, anemia, asthma, atherosclerosis, bronchitis, cholecystitis, Crohn's disease, ulcerative colitis, atopic dermatitis, dermatomyositis, diabetes mellitus, emphysema, erythema nodosum, atrophic gastritis, glomerulonephritis, gout, Graves' disease, hypereosinophilia, irritable bowel syndrome, lupus erythematosus, multiple sclerosis, myasthenia gravis, myocardial or pericardial inflammation, osteoarthritis, osteoporosis, pancreatitis, polymyositis, rheumatoid arthritis, scleroderma, Sjögren's syndrome, and autoimmune thyroiditis; complications of cancer, hemodialysis,

extracorporeal circulation; viral, bacterial, fungal, parasitic, protozoal, and helminthic infections; and trauma.

In another embodiment, a vector expressing the complement of the polynucleotide encoding PDE8 may be administered to a subject to treat or prevent an immune disorder including, but not limited to, those described above.

In another embodiment, an antagonist of PDE8 may be administered to a subject to prevent or treat a neuronal disorder. Such disorders may include, but are not limited to, akathesia, Alzheimer's disease, amnesia, amyotrophic lateral sclerosis, bipolar disorder, catatonia, cerebral neoplasms, dementia, depression, Down's syndrome, tardive dyskinesia, dystonias, epilepsy, Huntington's disease, multiple sclerosis, neurofibromatosis, Parkinson's disease, paranoid psychoses, schizophrenia, and Tourette's disorder.

In another embodiment, a vector expressing the complement of the polynucleotide encoding PDE8 may be administered to a subject to treat or prevent a neuronal disorder including, but not limited to, those described above.

In other embodiments, any of the proteins, antagonists, antibodies, agonists, complementary sequences or vectors of the invention may be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents for use in combination therapy may be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents may act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.

An antagonist of PDE8 may be produced using methods which are generally known in the art. In particular, purified PDE8 may be used to produce antibodies or to screen libraries of pharmaceutical agents to identify those which specifically bind PDE8.

Antibodies to PDE8 may be generated using methods that are well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, and fragments produced by a Fab expression library. Neutralizing antibodies, (i.e., those which inhibit dimer formation) are especially preferred for therapeutic use.

For the production of antibodies, various hosts including goats, rabbits, rats, mice, humans, and others, may be immunized by injection with PDE8 or any fragment or oligopeptide thereof which has immunogenic properties. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. Among adjuvants used in humans, BCG (bacilli Calmette-Guerin) and Corynebacterium parvum are especially preferable.

It is preferred that the oligopeptides, peptides, or fragments used to induce antibodies to PDE8 have an amino acid sequence consisting of at least five amino acids and more preferably at least 10 amino acids. It is also preferable that they are identical to a portion of the amino acid sequence of the natural protein, and they may contain the entire amino acid sequence of a small, naturally occurring molecule. Short stretches of PDE8 amino acids may be fused with those of another protein such as keyhole limpet hemocyanin and antibody produced against the chimeric molecule.

Monoclonal antibodies to PDE8 may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique (Kohler, G. et al. (1975) Nature 256:495-497; Kozbor, D. et al. (1985) J. Immunol. Methods 81:31-42; Cote, R.J. et al. (1983) Proc. Natl. Acad. Sci. 80:2026-2030; Cole, S.P. et al. (1984) Mol. Cell Biol. 62:109-120).

In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison, S.L. et al. (1984) Proc. Natl. Acad. Sci. 81:6851-6855; Neuberger, M.S. et al. (1984) Nature 312:604-608; Takeda, S. et al. (1985) Nature 314:452-454). Alternatively, techniques described for the production of single chain antibodies may be adapted, using methods known in the art, to produce PDE8-specific single chain antibodies. Antibodies with related specificity, but of distinct idiotypic composition, may be generated by chain shuffling from random combinatorial immunoglobulin libraries (Burton D.R. (1991) Proc. Natl. Acad. Sci.

88:11120-3).

Antibodies may also be produced by inducing in vivo production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature (Orlandi, R. et al. (1989) Proc. Natl. Acad. Sci. 86: 3833-3837; Winter, G. et al. (1991) Nature 349:293-299).

Antibody fragments which contain specific binding sites for PDE8 may also be generated. For example, such fragments include, but are not limited to, the F(ab')2 fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse, W.D. et al. (1989) Science 254:1275-1281).

Various immunoassays may be used for screening to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation between PDE8 and its specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering PDE8 epitopes is preferred, but a competitive binding assay may also be employed (Maddox, supra).

In another embodiment of the invention, the polynucleotides encoding PDE8, or any fragment or complement thereof, may be used for therapeutic purposes. In one aspect, the complement of the polynucleotide encoding PDE8 may be used in situations in which it would be desirable to block the transcription of the mRNA. In particular, cells may be transformed with sequences complementary to polynucleotides encoding PDE8. Thus, complementary molecules or fragments may be used to modulate PDE8 activity, or to achieve regulation of gene function. Such technology is now well known in the art, and sense or antisense oligonucleotides or larger fragments, can be designed from various locations along the coding or control regions of sequences encoding PDE8.

Expression vectors derived from retroviruses, adenovirus, herpes or vaccinia viruses, or from various bacterial plasmids may be used for delivery of nucleotide sequences to the

targeted organ, tissue or cell population. Methods which are well known to those skilled in the art can be used to construct vectors which will express nucleic acid sequence which is complementary to the polynucleotides of the gene encoding PDE8. These techniques are described both in Sambrook et al. (supra) and in Ausubel et al. (supra).

Genes encoding PDE8 can be turned off by transforming a cell or tissue with expression vectors which express high levels of a polynucleotide or fragment thereof which encodes PDE8. Such constructs may be used to introduce untranslatable sense or antisense sequences into a cell. Even in the absence of integration into the DNA, such vectors may continue to transcribe RNA molecules until they are disabled by endogenous nucleases. Transient expression may last for a month or more with a non-replicating vector and even longer if appropriate replication elements are part of the vector system.

As mentioned above, modifications of gene expression can be obtained by designing complementary sequences or antisense molecules (DNA, RNA, or PNA) to the control, 5' or regulatory regions of the gene encoding PDE8 (signal sequence, promoters, enhancers, and introns). Oligonucleotides derived from the transcription initiation site, e.g., between positions -10 and +10 from the start site, are preferred. Similarly, inhibition can be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules. Recent therapeutic advances using triplex DNA have been described in the literature (Gee, J.E. et al. (1994) In: Huber, B.E. and B.I. Carr, Molecular and Immunologic Approaches, Futura Publishing Co., Mt. Kisco, NY). The complementary sequence or antisense molecule may also be designed to block translation of mRNA by preventing the transcript from binding to ribosomes.

Ribozymes, enzymatic RNA molecules, may also be used to catalyze the specific cleavage of RNA. The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples which may be used include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of sequences encoding PDE8.

Specific ribozyme cleavage sites within any potential RNA target are initially

identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences: GUA, GUU, and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for secondary structural features which may render the oligonucleotide inoperable. The suitability of candidate targets may also be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays.

Complementary ribonucleic acid molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of nucleic acid molecules. These include techniques for chemically synthesizing oligonucleotides such as solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding PDE8. Such DNA sequences may be incorporated into a wide variety of vectors with suitable RNA polymerase promoters such as T7 or SP6. Alternatively, these cDNA constructs that synthesize complementary RNA constitutively or inducibly can be introduced into cell lines, cells, or tissues.

RNA molecules may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the backbone of the molecule. This concept is inherent in the production of PNAs and can be extended in all of these molecules by the inclusion of nontraditional bases such as inosine, queosine, and wybutosine, as well as acetyl-, methyl-, thio-, and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine which are not as easily recognized by endogenous endonucleases.

Many methods for introducing vectors into cells or tissues are available and equally suitable for use in vivo, in vitro, and ex vivo. For ex vivo therapy, vectors may be introduced into stem cells taken from the patient and clonally propagated for autologous transplant back into that same patient. Delivery by transfection, by liposome injections or polycationic amino polymers (Goldman, C.K. et al. (1997) Nature Biotechnology 15:462-66; incorporated herein by reference) may be achieved using methods which are well known in the art.

Any of the therapeutic methods described above may be applied to any subject in need

of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

An additional embodiment of the invention relates to the administration of a pharmaceutical composition, in conjunction with a pharmaceutically acceptable carrier, for any of the therapeutic effects discussed above. Such pharmaceutical compositions may consist of PDE8, antibodies to PDE8, mimetics, agonists, antagonists, or inhibitors of PDE8. The compositions may be administered alone or in combination with at least one other agent, such as stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone, or in combination with other agents, drugs or hormones.

The pharmaceutical compositions utilized in this invention may be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, or rectal means.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, PA).

Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars,

including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores may be used in conjunction with suitable coatings, such as concentrated sugar solutions, which may also contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, i.e., dosage.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a filler or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

Pharmaceutical formulations suitable for parenteral administration may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Non-lipid polycationic amino polymers may also be used for delivery. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes.

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder which may contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. For administration of PDE8, such labeling would include amount, frequency, and method of administration.

Pharmaceutical compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art.

For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays, e.g., of neoplastic cells, or in animal models, usually mice, rabbits, dogs, or pigs. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

A therapeutically effective dose refers to that amount of active ingredient, for example PDE8 or fragments thereof, antibodies of PDE8, agonists, antagonists or inhibitors of PDE8, which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from

cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors which may be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

Normal dosage amounts may vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

DIAGNOSTICS

In another embodiment, antibodies which specifically bind PDE8 may be used for the diagnosis of conditions or diseases characterized by expression of PDE8, or in assays to monitor patients being treated with PDE8, agonists, antagonists or inhibitors. The antibodies useful for diagnostic purposes may be prepared in the same manner as those described above for therapeutics. Diagnostic assays for PDE8 include methods which utilize the antibody and a label to detect PDE8 in human body fluids or extracts of cells or tissues. The antibodies may be used with or without modification, and may be labeled by joining them, either covalently or non-covalently, with a reporter molecule. A wide variety of reporter molecules which are known in the art may be used, several of which are described above.

A variety of protocols including ELISA, RIA, and FACS for measuring PDE8 are known in the art and provide a basis for diagnosing altered or abnormal levels of PDE8 expression. Normal or standard values for PDE8 expression are established by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with antibody to PDE8 under conditions suitable for complex formation. The amount of standard complex formation may be quantified by various methods, but preferably by photometric means. Quantities of PDE8 expressed in subject, control and disease samples from biopsied tissues are compared with the standard values. Deviation between standard and subject values establishes the parameters for diagnosing disease.

In another embodiment of the invention, the polynucleotides encoding PDE8 may be used for diagnostic purposes. The polynucleotides which may be used include oligonucleotide sequences, complementary RNA and DNA molecules, and PNAs. The polynucleotides may be used to detect and quantitate gene expression in biopsied tissues in which expression of PDE8 may be correlated with disease. The diagnostic assay may be used to distinguish between absence, presence, and excess expression of PDE8, and to monitor regulation of PDE8 levels during therapeutic intervention.

In one aspect, hybridization with PCR probes which are capable of detecting polynucleotide sequences, including genomic sequences, encoding PDE8 or closely related molecules, may be used to identify nucleic acid sequences which encode PDE8. The specificity of the probe, whether it is made from a highly specific region, e.g., 10 unique nucleotides in the 5' regulatory region, or a less specific region, e.g., especially in the 3' coding region, and the stringency of the hybridization or amplification (maximal, high, intermediate, or low) will determine whether the probe identifies only naturally occurring sequences encoding PDE8, alleles, or related sequences.

Probes may also be used for the detection of related sequences, and should preferably contain at least 50% of the nucleotides from any of the PDE8 encoding sequences. The hybridization probes of the subject invention may be DNA or RNA and derived from the nucleotide sequence of SEQ ID NO:2 or from genomic sequence including promoter, enhancer elements, and introns of the naturally occurring PDE8.

Means for producing specific hybridization probes for DNAs encoding PDE8 include

the cloning of nucleic acid sequences encoding PDE8 or PDE8 derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, commercially available, and may be used to synthesize RNA probes in vitro by means of the addition of the appropriate RNA polymerases and the appropriate labeled nucleotides. Hybridization probes may be labeled by a variety of reporter groups, for example, radionuclides such as 32P or 35S, or enzymatic labels, such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, and the like.

Polynucleotide sequences encoding PDE8 may be used for the diagnosis of conditions or disorders which are associated with expression of PDE8. Examples of such conditions or disorders include cancers, such as adenocarcinoma, leukemia, lymphoma, melanoma, myeloma, sarcoma, and teratocarcinoma, and, in particular, cancers of the adrenal gland, bladder, bone, bone marrow, brain, breast, cervix, gall bladder, ganglia, gastrointestinal tract, heart, kidney, liver, lung, muscle, ovary, pancreas, parathyroid, penis, prostate, salivary glands, skin, spleen, testis, thymus, thyroid, and uterus; immune disorders, such as AIDS, Addison's disease, adult respiratory distress syndrome, allergies, anemia, asthma, atherosclerosis, bronchitis, cholecystitis, Crohn's disease, ulcerative colitis, atopic dermatitis, dermatomyositis, diabetes mellitus, emphysema, erythema nodosum, atrophic gastritis, glomerulonephritis, gout, Graves' disease, hypereosinophilia, irritable bowel syndrome, lupus erythematosus, multiple sclerosis, myasthenia gravis, myocardial or pericardial inflammation, osteoarthritis, osteoporosis, pancreatitis, polymyositis, rheumatoid arthritis, scleroderma, Sjögren's syndrome, and autoimmune thyroiditis; complications of cancer, hemodialysis, extracorporeal circulation; viral, bacterial, fungal, parasitic, protozoal, and helminthic infections; and trauma; and neuronal disorders, such as akathesia, Alzheimer's disease, amnesia, amyotrophic lateral sclerosis, bipolar disorder, catatonia, cerebral neoplasms, dementia, depression, Down's syndrome, tardive dyskinesia, dystonias, epilepsy, Huntington's disease, multiple sclerosis, neurofibromatosis, Parkinson's disease, paranoid psychoses, schizophrenia, and Tourette's disorder. The polynucleotide sequences encoding PDE8 may be used in Southern or northern analysis, dot blot, or other membrane-based technologies; in PCR technologies; or in dipstick, pin, ELISA-like assays or microarrays utilizing fluids or tissues from patient biopsies to detect altered PDE8 expression. Such

qualitative or quantitative methods are well known in the art.

In a particular aspect, the nucleotide sequences encoding PDE8 may be useful in assays that detect activation or induction of various cancers, particularly those mentioned above. The nucleotide sequences encoding PDE8 may be labeled by standard methods, and added to a fluid or tissue sample from a patient under conditions suitable for the formation of hybridization complexes. After a suitable incubation period, the sample is washed and the signal is quantitated and compared with a standard value. If the amount of signal in the biopsied or extracted sample is significantly altered from that of a comparable control sample, the nucleotide sequences have hybridized with nucleotide sequences in the sample, and the presence of altered levels of nucleotide sequences encoding PDE8 in the sample indicates the presence of the associated disease. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or in monitoring the treatment of an individual patient.

In order to provide a basis for the diagnosis of disease associated with expression of PDE8, a normal or standard profile for expression is established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with a sequence, or a fragment thereof, which encodes PDE8, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified by comparing the values obtained from normal subjects with those from an experiment where a known amount of a substantially purified polynucleotide is used. Standard values obtained from normal samples may be compared with values obtained from samples from patients who are symptomatic for disease. Deviation between standard and subject values is used to establish the presence of disease.

Once disease is established and a treatment protocol is initiated, hybridization assays may be repeated on a regular basis to evaluate whether the level of expression in the patient begins to approximate that which is observed in the normal patient. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from several days to months.

With respect to cancer, the presence of a relatively high amount of transcript in biopsied tissue from an individual may indicate a predisposition for the development of the

disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type may allow health professionals to employ preventative measures or aggressive treatment earlier thereby preventing the development or further progression of the cancer.

Additional diagnostic uses for oligonucleotides designed from the sequences encoding PDE8 may involve the use of PCR. Such oligomers may be chemically synthesized, generated enzymatically, or produced in vitro. Oligomers will preferably consist of two nucleotide sequences, one with sense orientation (5'->3') and another with antisense (3'<-5'), employed under optimized conditions for identification of a specific gene or condition. The same two oligomers, nested sets of oligomers, or even a degenerate pool of oligomers may be employed under less stringent conditions for detection and/or quantitation of closely related DNA or RNA sequences.

Methods which may also be used to quantitate the expression of PDE8 include radiolabeling or biotinylating nucleotides, coamplification of a control nucleic acid, and standard curves onto which the experimental results are interpolated (Melby, P.C. et al. (1993) J. Immunol. Methods, 159:235-244; Duplaa, C. et al. (1993) Anal. Biochem. 212:229-236). The speed of quantitation of multiple samples may be accelerated by running the assay in an ELISA-like format where the oligomer of interest is presented in various dilutions and a spectrophotometric or colorimetric response gives rapid quantitation.

In further embodiments, oligonucleotides or longer fragments derived from any of the polynucleotide sequences described herein may be used as targets in a microarray. The microarray can be used to monitor the expression level of large numbers of genes simultaneously (to produce a transcript image), and to identify genetic variants, mutations and polymorphisms. This information may be used to determine gene function, to understand the genetic basis of disease, to diagnose disease, and to develop and monitor the activities of therapeutic agents.

In one embodiment, the microarray is prepared and used according to the methods known in the art such as those described in PCT application WO95/11995 (Chee et al.), Lockhart, D. J. et al. (1996; Nat. Biotech. 14: 1675-1680) and Schena, M. et al. (1996; Proc. Natl. Acad. Sci. 93: 10614-10619).

The microarray is preferably composed of a large number of unique, single-stranded nucleic acid sequences, usually either synthetic antisense oligonucleotides or fragments of cDNAs, fixed to a solid support. The oligonucleotides are preferably about 6-60 nucleotides in length, more preferably about 15 to 30 nucleotides in length, and most preferably about 20 to 25 nucleotides in length. For a certain type of microarray, it may be preferable to use oligonucleotides which are only 7 to 10 nucleotides in length. The microarray may contain oligonucleotides which cover the known 5' (or 3') sequence, or may contain sequential oligonucleotides which cover the full length sequence; or unique oligonucleotides selected from particular areas along the length of the sequence. Polynucleotides used in the microarray may be oligonucleotides that are specific to a gene or genes of interest in which at least a fragment of the sequence is known or that are specific to one or more unidentified cDNAs which are common to a particular cell or tissue type or to a normal, developmental, or disease state. In certain situations, it may be appropriate to use pairs of oligonucleotides on a microarray. The pairs will be identical, except for one nucleotide preferably located in the center of the sequence. The second oligonucleotide in the pair (mismatched by one) serves as a control. The number of oligonucleotide pairs may range from 2 to 1,000,000.

In order to produce oligonucleotides to a known sequence for a microarray, the gene of interest is examined using a computer algorithm which starts at the 5' or more preferably at the 3' end of the nucleotide sequence. The algorithm identifies oligomers of defined length that are unique to the gene, have a GC content within a range suitable for hybridization, and lack predicted secondary structure that may interfere with hybridization. In one aspect, the oligomers are synthesized at designated areas on a substrate using a light-directed chemical process. The substrate may be paper, nylon or any other type of membrane, filter, chip, glass slide, or any other suitable solid support.

In one aspect, the oligonucleotides may be synthesized on the surface of the substrate by using a chemical coupling procedure and an ink jet application apparatus, such as that described in PCT application WO95/251116 (Baldeschweiler et al.). In another aspect, a "gridded" array analogous to a dot or slot blot (HYBRIDOT apparatus, Life Technologies) may be used to arrange and link cDNA fragments or oligonucleotides to the surface of a substrate using a vacuum system, thermal, UV, mechanical or chemical bonding procedures.

In yet another aspect, an array may be produced by hand or by using available devices, materials, and machines (including multichannel pipettors or robotic instruments) and may contain 8, 24, 96, 384, 1536 or 6144 oligonucleotides, or any other multiple from 2 to 1,000,000 which lends itself to the efficient use of commercially available instrumentation.

In order to conduct sample analysis using the microarrays, polynucleotides are extracted from a biological sample. The biological samples may be obtained from any bodily fluid (blood, urine, saliva, phlegm, gastric juices, etc.), cultured cells, biopsies, or other tissue preparations. To produce probes, the polynucleotides extracted from the sample are used to produce nucleic acid sequences which are complementary to the nucleic acids on the microarray. If the microarray consists of cDNAs, antisense RNAs (aRNA) are appropriate probes. Therefore, in one aspect, mRNA is used to produce cDNA which, in turn and in the presence of fluorescent nucleotides, is used to produce fragment or oligonucleotide aRNA probes. These fluorescently labeled probes are incubated with the microarray so that the probe sequences hybridize to the cDNA oligonucleotides of the microarray. In another aspect, nucleic acid sequences used as probes can include polynucleotides, fragments, and complementary or antisense sequences produced using restriction enzymes, PCR technologies, and oligolabeling kits well known in the area of hybridization technology.

Incubation conditions are adjusted so that hybridization occurs with precise complementary matches or with various degrees of less complementarity. After removal of nonhybridized probes, a scanner is used to determine the levels and patterns of fluorescence. The scanned images are examined to determine degree of complementarity and the relative abundance of each oligonucleotide sequence on the microarray. A detection system may be used to measure the absence, presence, and amount of hybridization for all of the distinct sequences simultaneously. This data may be used for large scale correlation studies or functional analysis of the sequences, mutations, variants, or polymorphisms among samples (Heller, R.A. et al., (1997) Proc. Natl. Acad. Sci. 94:2150-55).

In another embodiment of the invention, the nucleic acid sequences which encode PDE8 may also be used to generate hybridization probes which are useful for mapping the naturally occurring genomic sequence. The sequences may be mapped to a particular chromosome, to a specific region of a chromosome or to artificial chromosome constructions,

such as human artificial chromosomes (HACs), yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), bacterial P1 constructions or single chromosome cDNA libraries as reviewed in Price, C.M. (1993) Blood Rev. 7:127-134, and Trask, B.J. (1991) Trends Genet. 7:149-154.

Fluorescent in situ hybridization (FISH as described in Verma et al. (1988) Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York, NY) may be correlated with other physical chromosome mapping techniques and genetic map data. Examples of genetic map data can be found in various scientific journals or at Online Mendelian Inheritance in Man (OMIM). Correlation between the location of the gene encoding PDE8 on a physical chromosomal map and a specific disease, or predisposition to a specific disease, may help delimit the region of DNA associated with that genetic disease. The nucleotide sequences of the subject invention may be used to detect differences in gene sequences between normal, carrier, or affected individuals.

In situ hybridization of chromosomal preparations and physical mapping techniques such as linkage analysis using established chromosomal markers may be used for extending genetic maps. Often the placement of a gene on the chromosome of another mammalian species, such as mouse, may reveal associated markers even if the number or arm of a particular human chromosome is not known. New sequences can be assigned to chromosomal arms, or parts thereof, by physical mapping. This provides valuable information to investigators searching for disease genes using positional cloning or other gene discovery techniques. Once the disease or syndrome has been crudely localized by genetic linkage to a particular genomic region, for example, AT to 11q22-23 (Gatti, R.A. et al. (1988) Nature 336:577-580), any sequences mapping to that area may represent associated or regulatory genes for further investigation. The nucleotide sequence of the subject invention may also be used to detect differences in the chromosomal location due to translocation, inversion, etc. among normal, carrier, or affected individuals.

In another embodiment of the invention, PDE8, its catalytic or immunogenic fragments or oligopeptides thereof, can be used for screening libraries of compounds in any of a variety of drug screening techniques. The fragment employed in such screening may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly.

The formation of binding complexes between PDE8 and the agent being tested may be measured.

Another technique for drug screening which may be used provides for high throughput screening of compounds having suitable binding affinity to the protein of interest as described in published PCT application WO84/03564. In this method, as applied to PDE8, large numbers of different small test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The test compounds are reacted with PDE8, or fragments thereof, and washed. Bound PDE8 is then detected by methods well known in the art. Purified PDE8 can also be coated directly onto plates for use in the aforementioned drug screening techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

In another embodiment, one may use competitive drug screening assays in which neutralizing antibodies capable of binding PDE8 specifically compete with a test compound for binding PDE8. In this manner, the antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants with PDE8.

In additional embodiments, the nucleotide sequences which encode PDE8 may be used in any molecular biology techniques that have yet to be developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, including, but not limited to, such properties as the triplet genetic code and specific base pair interactions.

The examples below are provided to illustrate the subject invention and are not included for the purpose of limiting the invention.

EXAMPLES

I cDNA Library Construction

THP1PLB02

The THP1PLB02 cDNA library was constructed by reamplification of THP1PLB01. The THP1PLB01 cDNA library was made from activated human monocytes by Stratagene (Stratagene, La Jolla, CA). Poly(A+)RNA was purified from THP-1 cells which were cultured for 48 hr with 100 nm TPA and activated with 1μg/ml LPS after 4 hr. cDNA

synthesis was primed separately with both oligo d(T) and random hexamers. Synthetic adaptor oligonucleotides were ligated onto cDNA ends enabling insertion into Uni-ZAP vector system (Stratagene). After construction, the two libraries were combined into a single library by mixing equal numbers of bacteriophage.

The cDNA library was screened with either DNA probes or antibody probes and the PBLUESCRIPT phagemid (Stratagene) was excised in vivo. The custom-constructed library phage particles were transfected into E. coli host strain XL1-BLUE cells (Stratagene). Alternative unidirectional vectors include, but are not limited to, pcDNAI (Invitrogen, San Diego, CA) and pSHlox-1 (Novagen, Madison, WI).

LATRNOT01

The LATRNOT01 cDNA library was obtained from left ventricle tissue from a 51 year-old Caucasian female (Lot No. RU95-03-196, IIAM).

The tissue was flash frozen and ground in a mortar and pestle. The tissue was lysed immediately in buffer containing guanidinium isothiocyanate and spun through cesium chloride. The precipitate was treated by several phenol chloroform extractions and ethanol precipitation at pH 8. The polyadenylated mRNA was then isolated, treated with DNase, and purified using OLIGOTEX mRNA purification kit (QIAGEN Inc., Chatsworth CA)

First strand cDNA synthesis was accomplished using an oligo d(T) primer/linker which also contained an XhoI restriction site. Second strand synthesis was performed using a combination of DNA polymerase I, E. coli ligase, and RNase H, followed by the addition of an EcoRI adaptor to the blunt ended cDNA. The EcoRI adapted, double-stranded cDNA was then digested with XhoI restriction enzyme and fractionated to obtain sequences which exceeded 800 bp in size. The cDNAs were inserted into the LAMBDAZAP vector system (Stratagene); and the vector which contained the PBLUESCRIPT phagemid (Stratagene) was transformed into E. coli host cells strain XL1-BLUEMRF cells (Stratagene).

II Isolation and Sequencing of cDNA Clones

THP1PLB02 and LATRNOT01

The phagemid forms of individual cDNA clones were obtained by the in vivo excision process, in which the host bacterial strain was coinfected with both the lambda library phage and an f1 helper phage. Polypeptides derived from both the library-containing phage and the helper phage nicked the lambda DNA, initiated new DNA synthesis from defined sequences on the lambda target DNA, and created a smaller, single stranded circular phagemid DNA molecule that included all DNA sequences of the PBLUESCRIPT plasmid and the cDNA insert. The phagemid DNA was secreted from the cells, purified, and used to re-infect fresh host cells, where the double stranded phagemid DNA was produced. Because the phagemid carries the gene for β-lactamase, the newly-transformed bacteria were selected on a medium containing ampicillin.

The THP1PLB02 phagemid DNA was purified using the MAGIC MINIPREPS DNA purification system (Promega, Madison, WI). The LATRNOT01 plasmid DNA was released from the cells and purified using the PREP 96-well plasmid purification kit. This kit consists of a 96-well block with reagents for 960 purifications. Alternatively, phagemid DNA was purified using the QIAWELL-8 Plasmid, QIAWELL PLUS, and QIAWELL ULTRA DNA purification system (QIAGEN, Chatsworth, CA). The DNA was eluted from the purification resin already prepared for DNA sequencing and other analytical manipulations.

The cDNAs were sequenced by the method of Sanger F and A.R. Coulson (1975; J Mol. Biol. 94:441f), using a MICROLAB 2200 liquid transfer system (Hamilton, Reno, NV) in combination with four PTC200 DNA ENGINE thermal cyclers (MJ Research, Watertown MA) and 377 or 373 DNA sequencing systems (PE Biosystems) and reading frame was determined.

III Homology Searching of cDNA Clones and Their Deduced Proteins

THP1PLB02

Each cDNA was compared to sequences in GenBank using a search algorithm developed by Applied Biosystems and incorporated into the INHERIT 670 sequence analysis system. In this algorithm, Pattern Specification Language (TRW Inc, Los Angeles, CA) was used to determine regions of homology. The three parameters that determine how the

sequence comparisons run were window size, window offset, and error tolerance. Using a combination of these three parameters, the DNA database was searched for sequences containing regions of homology to the query sequence, and the appropriate sequences were scored with an initial value. Subsequently, these homologous regions were examined using dot matrix homology plots to distinguish regions of homology from chance matches. Smith-Waterman alignments were used to display the results of the homology search.

Peptide and protein sequence homologies were ascertained using the INHERIT- 670 sequence analysis system using the methods similar to those used in DNA sequence homologies. Pattern Specification Language and parameter windows were used to search protein databases for sequences containing regions of homology which were scored with an initial value. Dot-matrix homology plots were examined to distinguish regions of significant homology from chance matches.

BLAST, which stands for Basic Local Alignment Search Tool (Altschul, S.F. (1993) J. Mol. Evol. 36:290-300; Altschul et al. (1990) J. Mol. Biol. 215:403-410), was used to search for local sequence alignments. BLAST produces alignments of both nucleotide and amino acid sequences to determine sequence similarity. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying homologs. BLAST is useful for matches which do not contain gaps. The fundamental unit of BLAST algorithm output is the High-scoring Segment Pair (HSP).

An HSP consists of two sequence fragments of arbitrary but equal lengths whose alignment is locally maximal and for which the alignment score meets or exceeds a threshold or cutoff score set by the user. The BLAST approach is to look for HSPs between a query sequence and a database sequence, to evaluate the statistical significance of any matches found, and to report only those matches which satisfy the user-selected threshold of significance. The parameter E establishes the statistically significant threshold for reporting database sequence matches. E is interpreted as the upper bound of the expected frequency of chance occurrence of an HSP (or set of HSPs) within the context of the entire database search. Any database sequence whose match satisfies E is reported in the program output.

LATRNOT01

The nucleotide sequences and/or amino acid sequences of the Sequence Listing were used to query sequences in the GenBank, SwissProt, BLOCKS, and Pima II databases. These databases, which contain previously identified and annotated sequences, were searched for regions of homology using BLAST, which stands for Basic Local Alignment Search Tool (Altschul, S.F. (1993) J. Mol. Evol 36:290-300; Altschul, et al. (1990) J. Mol. Biol. 215:403-410).

BLAST produced alignments of both nucleotide and amino acid sequences to determine sequence similarity. Because of the local nature of the alignments, BLAST was especially useful in determining exact matches or in identifying homologs which may be of prokaryotic (bacterial) or eukaryotic (animal, fungal, or plant) origin. Other algorithms such as the one described in Smith, T. et al. (1992, Protein Engineering 5:35-51), incorporated herein by reference, could have been used when dealing with primary sequence patterns and secondary structure gap penalties. The sequences disclosed in this application have lengths of at least 49 nucleotides, and no more than 12% uncalled bases (where N is recorded rather than A, C, G, or T).

The BLAST approach searched for matches between a query sequence and a database sequence. BLAST evaluated the statistical significance of any matches found, and reported only those matches that satisfy the user-selected threshold of significance. In this application, threshold was set at 10^{-25} for nucleotides and 10^{-10} for peptides.

Incyte nucleotide sequences were searched against the GenBank databases for primate (pri), rodent (rod), and other mammalian sequences (mam); and deduced amino acid sequences from the same clones were then searched against GenBank functional protein databases, mammalian (mamp), vertebrate (vrtp), and eukaryote (eukp) for homology.

IV Northern Analysis

Northern analysis is a laboratory technique used to detect the presence of a transcript of a gene and involves the hybridization of a labeled nucleotide sequence to a membrane on which RNAs from a particular cell type or tissue have been bound (Sambrook et al., supra).

Human multiple tissue northern blots (Clontech, Palo Alto, CA) were hybridized with

a probe consisting of the 5' most 939 nucleotides of clone 156196. Probe DNA was labeled with ^{32}P using the "Ready-To-Go" random prime labeling kit (Pharmacia Biotech Inc., Piscataway, NJ) and washed to a stringency of 0.5 x SSC, 65° C.

The highest levels of PDE8A were seen in testis, ovary, small intestine, and colon, but detectable levels were seen in heart, brain, placenta,lung, liver, skeletal muscle, kidney, pancreas, spleen, and prostate.

Computer techniques analogous to membrane based northern analysis were also performed using BLAST (Altschul (1993), supra; Altschul (1990), supra). The basis of the search is the product score which is defined as:

$$\frac{\% \text{ sequence identity} \times \% \text{ maximum BLAST score}}{100}$$

The product score takes into account both the degree of similarity between two sequences and the length of the sequence match. For example, with a product score of 40, the match will be exact within a 1-2% error; and at 70, the match will be exact. Homologous molecules are usually identified by selecting those which show product scores between 15 and 40, although lower scores may identify related molecules.

The results of northern analysis are reported as a list of libraries in which the transcript encoding PDE8 occurs. Abundance and percent abundance are also reported. Abundance directly reflects the number of times a particular transcript is represented in a cDNA library, and percent abundance is abundance divided by the total number of sequences examined in the cDNA library.

V Extension of PDE8 Encoding Polynucleotides

cDNA sequences containing 5' extensions of ESTs were extended by PCR amplification using human λgt10 testis or stomach cDNA libraries (Clontech Laboratories, Inc. Palo Alto, CA) and nested primers. For each reaction, 2.5 x 10^7 pfu were boiled for 5 minutes to release DNA. First round PCR (15 cycles) was performed with a PDE8A specific primer (8A specific-outer: 5'-GAAGCACATCAGCAGAAT-3'), (SEQ ID NO:10) and either a λgt10 forward (5'-TCGCTTAGTTTTACCGTTTTC-3', (SEQ ID NO:11), or a λgt10

reverse (5'-TATCGCCTCCATCAACAAACTT-3') (SEQ ID NO:12) primer. An aliquot, 1/50 of the reaction mixture, was used as a template for a second round of amplification (30 cycles) with a PDE8A specific primer (8A specific-inner: 5'-TTGTGGTAGGGATTGGAG-3') (SEQ ID NO:13) with either a nested λgt10 forward (5'-AGCAAGTTCAGCCTGGTTAAG-3') (SEQ ID NO:14), or λgt10 reverse (5'-CTTATGAGTATTTCTTCCAGGGTA-3') (SEQ ID NO:15) primer. Southern analysis of the PCR products used an internal PDE8A hybridization probe (5'-ATCATGGTTACAAATTATCGAAGCCAATTA-3') (SEQ ID NO:16). Positive bands were subcloned and sequenced. All sequences subsequently incorporated into the extended PDE8A sequence were verified by sequencing multiple independent PCR amplifications from the cDNA library DNA using unique primers, or by independent amplification from mRNA.

VI Labeling and Use of Individual Hybridization Probes

Hybridization probes derived from polynucleotide sequences of the invention are employed to screen cDNAs, genomic DNAs, or mRNAs. Although the labeling of oligonucleotides, consisting of about 20 base-pairs, is specifically described, essentially the same procedure is used with larger nucleotide fragments. Oligonucleotides are designed using state-of-the-art software such as OLIGO 4.06 software (National Biosciences), labeled by combining 50 pmol of each oligomer and 250 μCi of [γ-^{32}P] adenosine triphosphate (Amersham Pharmacia) and T4 polynucleotide kinase (NEN Life Science Products, Boston, MA). The labeled oligonucleotides are substantially purified with Sephadex G-25 superfine resin column (Amersham Pharmacia). A aliquot containing 10^7 counts per minute of the labeled probe is used in a typical membrane-based hybridization analysis of human genomic DNA digested with one of the following endonucleases Ase I, Bgl II, Eco RI, Pst I, Xba 1, or Pvu II; (NEN Life Science Products, Boston, MA).

The DNA from each digest is fractionated on a 0.7 percent agarose gel and transferred to NYTRAN PLUS nylon membranes (Schleicher & Schuell, Durham, NH). Hybridization is carried out for 16 hours at 40°C. To remove nonspecific signals, blots are sequentially washed at room temperature under increasingly stringent conditions up to 0.1 x saline sodium citrate and 0.5% sodium dodecyl sulfate. After XOMAT AR film (Eastman Kodak,

Rochester, NY) is exposed to the blots in a PHOSPHORIMAGER cassette (Molecular Dynamics, Sunnyvale, CA) for several hours, hybridization patterns are compared visually.

VII Microarrays

To produce oligonucleotides for a microarray, one of the nucleotide sequences of the present invention are examined using a computer algorithm which starts at the 3' end of the nucleotide sequence. The algorithm identified oligomers of defined length that are unique to the gene, have a GC content within a range suitable for hybridization, and lack predicted secondary structure that would interfere with hybridization. The algorithm identifies approximately 20 sequence-specific oligonucleotides of 20 nucleotides in length (20-mers). A matched set of oligonucleotides are created in which one nucleotide in the center of each sequence is altered. This process is repeated for each gene in the microarray, and double sets of twenty 20 mers are synthesized and arranged on the surface of the silicon chip using a light-directed chemical process, such as that discussed in Chee, supra.

In the alternative, a chemical coupling procedure and an ink jet device are used to synthesize oligomers on the surface of a substrate (cf. Baldeschweiler, supra). In another alternative, a "gridded" array analogous to a dot (or slot) blot is used to arrange and link cDNA fragments or oligonucleotides to the surface of a substrate using a vacuum system, thermal, UV, mechanical or chemical bonding procedures. A typical array may be produced by hand or using available materials and machines and contain grids of 8 dots, 24 dots, 96 dots, 384 dots, 1536 dots or 6144 dots. After hybridization, the microarray is washed to remove nonhybridized probes, and a scanner is used to determine the levels and patterns of fluorescence. The scanned image is examined to determine degree of complementarity and the relative abundance/expression level of each oligonucleotide sequence in the microarray.

VIII Complementary Polynucleotides

Sequences complementary to the PDE8-encoding sequence, or any part thereof, are used to decrease or inhibit expression of naturally occurring PDE8. Although use of oligonucleotides comprising from about 15 to about 30 base-pairs is described, essentially the same procedure is used with smaller or larger sequence fragments. Appropriate

oligonucleotides are designed using OLIGO 4.06 software (National Biosciences) and the coding sequence of PDE8. To inhibit transcription, a complementary oligonucleotide is designed from the most unique 5' sequence and used to prevent promoter binding to the coding sequence. To inhibit translation, a complementary oligonucleotide is designed to prevent ribosomal binding to the PDE8-encoding transcript.

IX Expression of PDE8

A 1.6 kb region of PDE8A(E) (the C-terminal 545 amino acids) was amplified and cloned into the baculovirus transfer vector pFASTBAC (Life Technologies, Inc., Gaithersburg, MD), which had been modified to include a 5' FLAG tag. Recombinant virus stocks were prepared according to the manufacturer's protocol. Sf9 cells were cultured in Sf900 II Sfm serum free media (Life Technologies Inc.) at 27°C. For expression, 1 x 10^8 Sf9 cells were infected at a multiplicity of infection of 5 in a final volume of 50 mls. At three days post-infection, the cells were harvested, and enzyme-containing lysates were prepared as detailed below. To monitor expression, 1 ml each of mock-infected and PDE8A(E) infected cell lysate was electrophoresed in a polyacrylamide gel and either silver-stained by standard methods or transferred to nitrocellulose and assayed using western analysis and an anti-FLAG antibody (M2, Scientific Imaging System, Eastman Kodak, New Haven, CT) at a concentration of 2 mg/ml. The secondary antibody was an alkaline phosphatase conjugated anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN) and the blot was visualized with a "BCIP/NBT phosphatase substrate system" (Kirkegaard & Perry Laboratories, Gaithersburg , MD) according to the manufacturer's protocol.

PDE8A(E) to be used for assay was prepared from transfected Sf9 cells. Cells were harvested by centrifugation, resuspended in homogenization buffer (20 mM Tris-HCl, 2 mM benzamidine, 1mM EDTA, 0.25 M sucrose, 100 uM PMSF, pH 7.5) at 1x10^7 cells/ml, and disrupted using a Branson sonicating probe (3 x10 second pulses). Cellular debris was removed by centrifugation at 14,000xg for 10 minutes. The supernatant was stored at -70°C.

X Demonstration of PDE8 Activity

PDE activity was assayed by measuring the conversion of ^{3}H-cAMP to ^{3}H-adenosine

in the presence of PDE8A(E) and 5' nucleotidase. A one-step assay was run using a 100 uL assay containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl^2$, 0.1 unit 5'nucleotidase (from Crotalus atrox venom), 0.0062-0.1 uM ^{3}H- cAMP, and various concentrations of cAMP (0.0062-3 mM). The reaction was started by the addition of 25 ul of diluted enzyme supernatant. Reactions were run directly in mini Poly-Q scintillation vials (Beckman Instruments Inc., Fullerton CA). Assays were incubated at 37°C for a time period that would give less than 15% cAMP hydrolysis to avoid non-linearity associated with product inhibition. The reaction was stopped by the addition of 1ml of Dowex AG1x8 (Cl form) resin (1:3 slurry). Three ml of scintillation fluid were added, and the vials were mixed. The resin in the vials was allowed to settle for 1 hr before counting. Soluble radioactivity associated with ^{3}H-adenosine was quantitated using a Beta scintillation counter. The amount of radioactivity recovered is proportional to the activity of PDE8 in the reaction. For inhibitor studies (Fig. 8), all reactions contained 1% DMSO, 50 nM cAMP, and the indicated inhibitor concentrations. The control vial contained all reagents minus the enzyme aliquot.

XI Production of PDE8 Specific Antibodies

PDE8 that is substantially purified using PAGE electrophoresis (Sambrook, supra), or other purification techniques, is used to immunize rabbits and to produce antibodies using standard protocols. The amino acid sequence deduced from SEQ ID NO:2 is analyzed using LASERGENE software to determine regions of high immunogenicity and a corresponding oligopeptide is synthesized and used to raise antibodies by means known to those of skill in the art. Selection of appropriate epitopes, such as those near the C-terminus or in hydrophilic regions, is described by Ausubel et al. (supra), and others.

Typically, the oligopeptides are 15 residues in length, synthesized using an ABI 431A peptide synthesizer (PE Biosystems) using fmoc-chemistry, and coupled to keyhole limpet hemocyanin (KLH, Sigma, St. Louis, MO) by reaction with N-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Ausubel et al., supra). Rabbits are immunized with the oligopeptide-KLH complex in complete Freund's adjuvant. The resulting antisera are tested for antipeptide activity, for example, by binding the peptide to plastic, blocking with 1% BSA, reacting with rabbit antisera, washing, and reacting with radio iodinated, goat anti-

rabbit IgG.

XII Purification of Naturally Occurring PDE8 Using Specific Antibodies

Naturally occurring or recombinant PDE8 is substantially purified by immunoaffinity chromatography using antibodies specific for PDE8. An immunoaffinity column is constructed by covalently coupling PDE8 antibody to an activated chromatographic resin, such as CNBr-activated SEPHAROSE (APB). After the coupling, the resin is blocked and washed according to the manufacturer's instructions.

Media containing PDE8 is passed over the immunoaffinity column, and the column is washed under conditions that allow the preferential absorbance of PDE8 (e.g., high ionic strength buffers in the presence of detergent). The column is eluted under conditions that disrupt antibody/PDE8 binding (eg, a buffer of pH 2-3 or a high concentration of a chaotrope, such as urea or thiocyanate ion), and PDE8 is collected.

XIII Identification of Molecules Which Interact with PDE8

PDE8 or biologically active fragments thereof are labeled with ^{125}I Bolton-Hunter reagent (Bolton et al. (1973) Biochem. J. 133: 529). Candidate molecules previously arrayed in the wells of a multi-well plate are incubated with the labeled PDE8, washed and any wells with labeled PDE8 complex are assayed. Data obtained using different concentrations of PDE8 are used to calculate values for the number, affinity, and association of PDE8 with the candidate molecules.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.